Review Paper:

CRISPR/Cas gene editing based therapy approach in clinical therapeutics

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Abstract

Advanced inventions in genetic engineering towards clinical therapeutics have been rising over the past decade, which has brought a new approach in treating diseases like HIV, cancer, pulmonary diseases, genetic disorders, hereditary transferring disorders etc. Gene editing-based therapy holds specific molecular tools i.e. ZFN, TALENS and CRISPR/Cas9. These gene-editing techniques show precise and accurate modification of the eukaryotic genome. The application of CRISPR/Cas had become a trending gene-editing technique as its effectiveness in editing was comparatively high. Nowadays, gene-editing based therapy includes gene therapy approaches i.e. in vivo and ex vivo strategies, to reach efficient clinical treatment requirements. A new strategy of editing was made by assigning ss-ODN along with CRISPR, TALENS and ZFN, which can also increase the efficiency of gene editing, leading to promising results.

In this review, we discussed advanced gene-editing techniques and few recent implementations of genome editing to combat several diseases and disorders along with their effective clinical strategies. We also added a few CRISPR/Cas9-developed animal models that are used in treating human genetic disorders.

Keywords: CRISPR/Cas9, ssODNs, Genetic engineering, Gene editing, Gene therapy, Clinical therapeutics.

Introduction

In recent scenarios, the development of genome-editing techniques in clinical therapeutics has been relatively high. The genome editing approach showed an enlightening way of treating various infectious diseases, hereditary transferring infections, neoplastic diseases and a few genetic disorders. Gene editing involves either adding or removing a transgene that can be stable and inducible, which replaces the malfunctioning gene with the help of molecular tools to produce a disease-free or corrected genome used to treat diseases. This is known as gene therapy. The key objective of using gene editing in clinical settings is to treat diseases[16,65].

Restrictions enzymes known as nucleases have DNA-binding domains that induce double-strand breaks in the genome, which allows for site-specific editing[50,71]. There are three effective nucleases in genetic engineering: ZFN's, TALEN's and CRISPR/Cas9[35,68,76]. Generally, gene therapy strategies are mainly based on two approaches: *in vivo* and *ex vivo*. *In vivo* approaches involve a new functional gene with carrier vectors like plasmids or any viral vectors that drive into the patient's body. This therapy enables the delivery of transgene into patients' bodies either systematically or locally to body organs like the eyes, liver, brain and skeletal muscle.

The *ex-vivo* gene therapy approach involves removing specific cells from the body of the patient, genetically altering them and then replacing target cells that have a missing or malfunctioning gene[36]. TALENs, CRISPR/Cas9 gene editing and zinc finger nucleases are considered as the most effective gene therapy tools[43,56]. A broad spectrum of neurological disorders such as Huntington's disease (HD), stroke, spinal cord injury (SCI), traumatic brain injuries, Parkinsonism, Alzheimer's, Werdnig-Hoffmann disease, myotrophic lateral sclerosis (ALS), epilepsy, neurological conditions linked to COVID-19, various cancers, HIV infections and other infectious diseases, are treated with them[22,44,58].

Contrasting the three gene editing approaches, although TALENS and ZFNs have previously shown precise gene editing, the CRISPR/Cas system has gained appeal in recent years because of its accurate and efficient gene editing, capabilities and because it is trusted as one of the safest and most effective delivery methods in clinical applications[30]. All three types of gene editing have benefits and drawbacks, but the peculiarity of the disease or damage that should be altered, reveals a preference for particular gene-editing technique. In this review, brief overview of CRISPR/Cas gene-editing and different approaches to treat genetic and clinical disorders is discussed. We also covered some novel ways to use the CRISPR/Cas system to create animal models that are useful for a range of clinical applications.

***CRISPR-Cas* System:** The prokaryotic genome contains palindromic sequences termed Clustered Regularly Interspaced Short Palindromic Repeats, which function as an antiviral defense mechanism and offer a type of acquired immunity[4]. Using CRISPR sequences as a guide, the enzyme Cas9 identifies locations and cuts strands of DNA that are complementary to the CRISPR sequence (Figure 1). Cas9 enzymes and CRISPR sequences work together to alter genes within organisms, an approach known as CRISPR[33,84]. There are two main classes in the CRISPR-Cas system: class 1 and class 2. The class 1 CRISPR system is linked to several

Cas proteins that aid in pre-crRNA processing, binding and cleavage-induced interference. It is probable possible to categorize class 1 into three types: type I, III and IV.

Recent developments in computing have revealed that the CRISPR system has 33 new variants in the class 1 category. Class 2, which binds Cas proteins, is a single multidomain crRNA[51]. Cas proteins which target both RNA and DNA, comprise this system, which is further subdivided into types II, V and VI[2, 72]. A CRISPR/Cas system called *Sp*Cas9 was first employed to modify human cell genomes. It was identified in *Streptococcus pyogenes* and it encodes DNA targeting ability at its first 20 nucleotides. It guides RNA that is approximately 100 nucleotides long. *Sp*Cas9 unwinds DNA and ends at the NGG sequence (where 'N' means any nucleotide). It then assembles the spacer of the guide RNA opposite to the strand of DNA, which has no NGG sequence. Strong complementarity between the target strand and spacer region causes binding between DNA-RNA molecules, which in turn activates *Sp*Cas9, causing a double-strand break near the third base pairing upstream of the NGG sequence.

The protospacer, a 20-nucleotide sequence located adjacent to the NGG nucleotide, is the sequence on the non-target strand that corresponds to the RNA spacer sequence. There are a few examples of other kinds of bacteria that also contain Cas9 proteins, which are also employed in genome editing. One such example is the *Staphylococcus aureus* Cas9 (*Sa*Cas9) protein, which differs from *Sp*Cas9 in its PAM sequence, allowing for a distinct targeting range[64]. Protein engineering has produced various new *Sp*Cas9 and *Sa*Cas9 variants that can identify unique PAM sequences. In human cells and bacterial selection systems, *Staphylococcus aureus* Cas9 (*Sa*Cas9) and *Streptococcus thermophilus* Cas9 (*St*1Cas9) both work effectively[39]. Members of the Cas12 family, namely Cas12b/C2c1, Cas12a/Cpf1 and Cas12e/CasX, are effective genome editors[47,74].

Moreover, it has been discovered that the CRISPR/Cas9 system can work when additional domains are fused to the dCas9 or nCas9 proteins. Reverse transcriptase and nCas9 can be used to generate a DNA strand complementary to a single-strand RNA substrate. To create a sequence of RNA matching the non-target DNA, the guide RNA stretches over its substrate at both 3′ ends. Adenine base editors (ABE's) and cytosine base editors (CBE's) are two important kinds of base editors. CBE can change the base C on a DNA strand to another base (usually T, but occasionally G) whereas adenine base editors can change the base A to G[24,40,41]. Although the CRISPR-Cas9, Cas12 and Cas13 systems contain RNA and components of proteins, their actions are limited to target RNAs, comparatively targeting DNAs. RNA editors can be employed to modify target RNAs by adding base edits (such as A-to-I or C-to-U changes) or to destroy target RNAs[1,13]. In hemophilia treatment, LNP-mediated CRISPR-Cas9 administration was found to be a safe and effective method, as it demonstrated AT inhibition that enhanced thrombin production[27]. Repetitive intramuscular injections of Cas9 mRNA and sgRNA are delivered into skeletal muscle via the lipid nanoparticle (LNP) system. This method's reduced immunogenicity and repeated administration make it a promising gene-transferring vehicle for CRISPR-Cas9 in the treatment of Duchenne muscular dystrophy[37]. A single intracerebral injection of CRISPR-LNPs against PLK1 (sgPLK1-cLNPs) was administered to treat aggressive orthotopic glioblastoma. This approach led to around 70% gene editing *in vivo*, a 50% decrease in tumor growth and a 30% survival rate.

In addition to inhibiting tumor development, the intraperitoneal administration of EGFR-targeted sgPLK1-cLNPs in disseminated ovarian tumors led to 80% enhanced survival and up to ~80% gene editing *in vivo*[66]. Cas9-based medicines can be delivered safely and effectively with LNP-mediated delivery. Lipid nanoparticle delivery containing Cas9 messenger and guide RNA was created for Angptl3 genome editing using CRISPR/Cas9. Angiopoietin-like 3 (Angptl3) loss-of-function mutations are linked to decreased blood lipid levels and Angptl3 was a potential target for treating human lipoprotein metabolism disorders[62]. Numerous liver-related disorders can be effectively treated using LLN-mediated CRISPR/Cas9 delivery[32,55].

Using gene editing techniques like ribonucleoprotein complexes of Cas9, Cas9 mRNA guides and RNA selective organ targeting (SORT) facilitates the creation of gene correction and protein replacement[11]. In cancer immunotherapy, programmed death-ligand (PD-L1) inhibitors are a common target and are widely used. CRISPR/Cas9 is employed to reduce PD-1 expression in metastatic cells from patients with non-small cell lung cancer (NSCLC) [14]. Targeting PD-1 expression in T-cells was practiced in cancers of the renal, bladder and prostate cells[75].

Through the CRISPR-Cas9 approach in T cells for individuals with resistant cancer, antitumor immunity improvement was observed by deleting both genes encoding the endogenous T cell receptor (TCR) chains, TCRα (TRAC) and TCRβ (TRBC), within T cells to decrease TCR mispairing and to enhance the expression of a synthetic, cancer-specific TCR transgene (NY-ESO-1). A transcript for programmed cell death protein 1 is produced when a third gene is deleted[73]. The CRISPR/Cas system was employed to treat several hereditary disorders in children including lymphoma, neuroblastoma, sickle cell anemia, thalassemia and mucopolysaccharidosis type IVA[5,15,25,60,61,67,77].

When several targeted RTK/Ras/MAPK pathway inhibitors are present, deletion of KEAP1 alters cell metabolism by enabling cells to grow without MAPK signaling, which will help to determine the effectiveness of relevant medications in clinical trials and direct treatment choices. Thus, CRISPR/Cas9 was also employed for lung cancer cell

screening, specifically for CRISPR/Cas9 gene deletion screening[42]. CRISPR/Cas9 also holds effective nanoscale delivery of phytochemicals, which shows intense hope in cancer therapy[25]. In CRISPR-based technology, RNA-guided Cas13 was known to recognize the genetic features of SARS-Coronavirus-2 and to demonstrate a unique coronavirus suppression technique. SHERLOCK and DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) methods have been introduced as SARS-CoV-2 diagnostic tools, offering a more sophisticated method than CRISPR/Cas-mediated virus identification[30,63].

Lentiviral particles containing mRNA were employed to specifically target HSV-1 genomes, delivering *Sp*Cas9 mRNA and viral guide RNAs that target genes. It was demonstrated that the viral reservoir can be eliminated by HSV-1-deleting lentiviral particles through retrograde transmission from the corneas to the trigeminal ganglia. It draws attention to the therapeutic potential of HELP in the management of refractory HSK[82].

Forward Step of CRISPR/cas9 in creating Animal Models to treat Human Genetic Disorders: Apolipoprotein-B editing complex-1, activation-induced deaminase and cytidine deaminases were the variants of Cas9 that were successfully fused with nCas9 to create cytidine base editor (BE) systems. A modified tRNA adenosine deaminase i.e. ecTadA from Escherichia coli[26, 48]. is combined with nCas9 to create adenine base editors (ABEs), which convert A-T base pairs in genomic DNA to G-C. Cas9 adenosine base editors (Cas9-ABEs) and Cas9 cytidine base editors (Cas9-CBEs) can modify a gene without causing a double strand break by simply adding premature stop (pmSTOP) codons or deactivating splice sites (BE-splice). It has recently been shown that the CRISPR/Cas9 technology allows targeted A-to-G7 (ABE7.10) or C-to-T4,5,6 (BE3) conversion in living cells without requiring template-donor DNA or generating double-strand breaks.

The targeted mutation efficacy in the BE3 and ABE7.10 approaches was 53-88% in blastocysts and 44-100% in Founder (F0) rabbits. Rabbit animal models that mimic the human Hutchinson-Gilford progeria syndrome (HGPS) have been successfully generated with the CRISPR/Cas base editor. People with HGPS, a rare genetic condition caused by a pG608G *de novo* point mutation in the lmna gene, age quicker than others. It was also found to display the same phenotype as human HGPS which includes growth retardation, low stature, deformities in the bones and loss of subcutaneous fat, by inserting a point mutation in the rabbit-Lmna gene.

This suggests that the CRISPR/Cas rabbit HGPS model can be used to accurately represent the genetic illness linked to human HGPS[48]. A mutation in acid alpha-glucosidase (Gaa) causes an accumulation of glycogen-filled lysosomes in tissues which results in skeletal muscle and heart failure. Base-edited rodents and mice were created to imitate human diseases using CRISPR/Cas ABEs, which had a maximum efficiency of up to 100%. The ABE approach is a commanding and useful tool for demonstrating accurate base adaptations in rodents[81]. Androgen receptors (Ar) and homeobox D13 (Hoxd13) have been associated with syndactyly and androgen insensitivity syndrome (AIS), In these mouse models with clinically significant mutations at Ar and Hoxd13, which recapitulate the related clinical problems, cytosine to thymine and adenine to guanine base editing is possible using ABE and SaBE3[49]. Recently, base editors have been used more often, successfully producing missense mutations and early stop codons, such as the third-generation base editor (BE3). Targeting the TWIST2 and TYR genes, BE3 was able to produce pig models that closely resembled the phenotypes of human diseases. For instance, the ablepharon macrostomia syndrome (AMS), which is caused by the TWIST2 gene, causes severe malformations like macrostomia, microtia and absent eyelids.

Ocular-cutaneous albinism type 1 is caused by the TYR gene (OCA1)[46]. Using CRISPR/Cas9, the R124C mutation in TGFBI was created to create a unique transgenic animal:mice with a single amino acid substitution of cysteine for arginine 124 in TGFBI through ssODN-mediated base-pair substitution, which results in Lattice Corneal Dystrophy Type 1 (LCD1)[38]. The allotetraploid *X. laevis* (African clawed frog) had rhodopsin-encoding genes. CRISPR/Cas9 technology was extended to make targeted insertions, knockout alleles and dominant alleles. The use of *X. laevis*, an experimental organism, for biological study and disease modeling is increased by high-frequency gene editing. High-frequency editing of *X. laevis* genes was a success in the experiment, including F0 generation analysis[19].

ssOND (Short Synthetic Oligodeoxynucleotides Associated Gene Editing): Using synthetic oligodeoxynucleotides and small site-specific gene modifications, short synthetic oligodeoxynucleotide-associated gene editing is applied to alter the genomic DNA. This ssOND complements the target genomes at a specific location[59] 2'-O-methylated uracil bases and phosphothioate-conjugates are often used to produce modified ssODNs with excellent stability through resistance[18,31]. The ability of the ssODN-associated CRISPR/Cas system to alter genes was well understood[53]. One of the most successful approaches to gene editing is the use of ssODNs to fix mutations using the CRISPR-Cas9 method for the creation of gene therapy to treat genetic health diseases[78].

ssODNs have developed the technology into a reproducible tool with therapeutic potential[57]. SS ODNs are coupled to TALENs to achieve effective gene editing by annealing to their complementary nucleotide sequence at the target location and serving as primers for replication fork extension. Corrected cells fail to multiply in a condition known as the Reduced Proliferation Phenotype (RPP).

To overcome this drawback, TALENs are used to improve proliferation frequency by reducing the ssODN essential to direct gene correction. This approach resolves the issue and stops RPP's negative consequences. Significant amounts of ssODN molecules were given to the cells to affect the genetic change. By focusing on cells that are in the S phase of cell division, gene editing can be made much more effective. The efficacy of HDR significantly enhanced gene mutation alteration at both the β-catenin Ser45 deletion mutation cells and the GFP-silent mutation cells, compared to the effectiveness achieved by homology-directed repair (HDR) with ssODNs templates by combining with CRISPR/Cas9, eGFP and DNA ligase IV inhibitor SCR7.

When transfected cells were treated with SCR7, the targeted insertion efficiency increased threefold over control cells[28].

Comparable to this, an animal study of OTOF mutations that induce homology-directed repair through linking the CRISPR system with various Cas9 proteins in combination with ssODN, could contribute to developing new treatments and improving scientific understanding of genetic disorders related to deafness in humans[61]. ssODN and Cas9 RNPs demonstrated a better degree of correction than Cas12a in the CFF-16HBEge W1282X CFTR cell line (obtained from CFF). Around 18% of precise editing had been achieved compared to merely 8% for Cas12a. To repair the W1282X CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) mutation, HDR acted as a mediator[69]. It is also reported that synergistic gene editing, or integrating ssODN repair templates with biallelic HDR to produce heterozygous mutations, is an efficient method for producing precise genetic alterations in human iPS cells[52].

Table 1
Recent pre-clinical development of CRISPR -Cas

Organ	**Disease**	**Target gene and Editing**
Lung	Metastatic non-small-cell lung cancer	PD-1 gene in T cells from patient peripheral blood via sgRNA and Cas9 plasmid electroporation[14]
Blood	Refractory cancer	PDCD1 (encoding PD-1 loci) and TRAC and TRBC genes, which encode the chains of endogenous TCR, were removed from the T cells that were taken from the patients in order to boost anti-tumor immunity[73].
Blood/ Bone marrow	Refractory hematological malignancies	To generate gene-disrupted allogeneic global CD19-specific CAR T cells (UCART019), two CARs (i.e., CD19 and CD20 or CD22) were integrated into the TRAC locus of T cells, which were able to identify CD19− cells. CARs were delivered using lentivirus (LV) [20].
Liver	Hypercholesteolemia	• BE3, disruption of mouse W159 Pcsk9[9]. • BE3, disruption of human W159 PCSK10 or NHEJ-mediated disruption of PCSK10[7].
Muscle	DMD, exon 50 DMD, exon 44	Reframing or Exon skipping[3, 54]
Ear	Dominant progressive hearing loss (DFN36), TMC1	Knockdown mutant Tmc1[23]
Liver	Atherogenic dyslipidemia	BE3, disruption of Q135 Angptl3[8]
Liver	Hereditary tyrosinemia type I, autosomal recessive, FAH loss of function mutation	HDR[83,70]
Liver	Hemophilia A, X-linked recessive	Insert human B-domain deleted FVIII in intron 13 of liver-specific albumin locus[10]
Liver	Hemophilia B, X-linked recessive	HDR, insert hFIX-padua exon 2–8 in mFIX exon 2[79]
Neurological Disorder	Huntington's disease (CAG trinucelotide), expansion mutant HT	Knockdown HTT/ Knockdown mutant HTT[78, 17]
Neurological Disorder	Fragile X syndrome	Knockdown GRM5[45]
Neurological Disorder	Dravet Syndrome, haploinsufficiency, SCN1A loss of function mutation	dCas9-Vp64 mediated Scn1a gene activation (both WT and mutant)[12]
Neurological Disorder	Alzheimer's disease	Knockdown Bace1[60]
Limbs	knocking out miRNA-155	rheumatoid arthritis[34]
Ovary	ovarian cancer	knock down the expression of pre-miR-21[29]
Eye	Retinitis pigmentosa, autosomal recessive PDE6B mutation	SpCas9/Rec A mediated HDR[6]

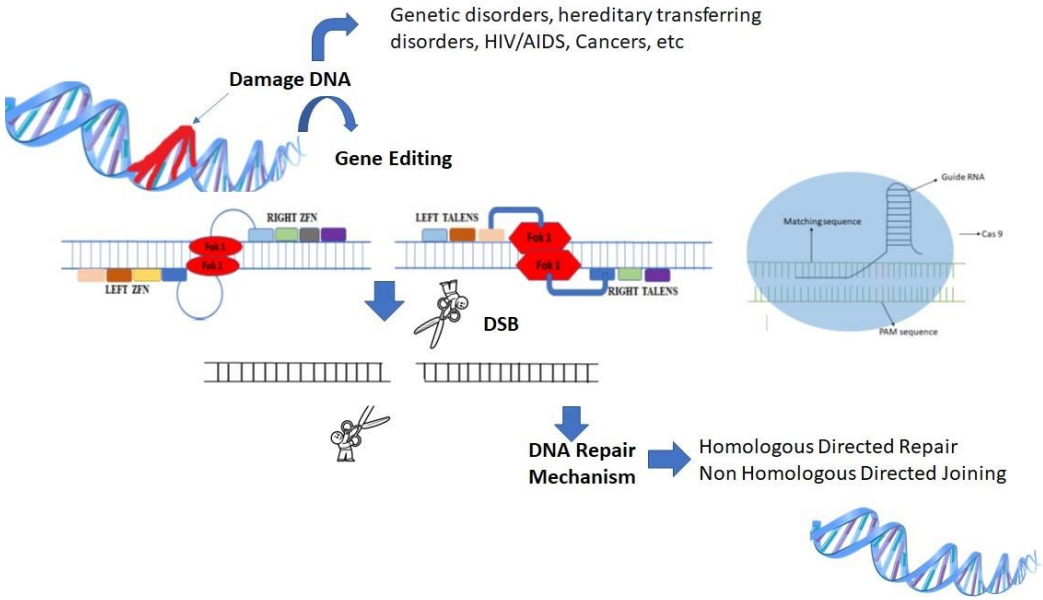

Figure 1: Approach of Zinc Fingers, TALENS and CRISPR/Cas gene editing to treat clinical disorders or diseases.

Conclusion

Genome editing technology is currently growing so much in clinical medicine to treat various diseases. As few diseases had no cure via drug intake or following any medical procedures, genome editing in therapeutics showed a paving way to treat those diseases by correction of specific genes by either knock-out or knock-in mechanisms at specific gene sequences and many trustful practices had proven the efficacy of gene therapy. Collaborating programmed gene-altering systems with specific molecular tools like single-stranded oligodeoxynucleotides (ssODN) show effective gene therapy clinically, which can help patients to get rid of a few deadly diseases.

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